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The changes in the gene program of neoplastic cells that regulate the expression of an invasive phenotype are largely undefined. Direct comparisons of the gene expression profile displayed in normal and carcinomatous breast tissues have provided insights into the mechanisms underlying tumor progression. However, attempts to identify the gene products differentially expressed during invasion *in vivo* have been hampered by the fact that only a small percentage of the cells recovered from a tumor mass are actively engaged in invasive behavior at the time of isolation. Because tissue remodeling induced during mammary gland involution bears homology to early stages of carcinogenesis, the involuting mammary gland may be used to identify genes that control matrix turnover in cancerous states. To this end, we propose to *i) generate cDNA libraries from control versus involuting mouse mammary glands, ii) isolate differentially expressed genes during matrix remodeling, iii) identify differentially expressed genes that encode secretory proteins associated with the involution program and iv) identify human homologues of the mouse-derived matrix remodeling genes.* The approach should allow for the identification of gene products relevant to breast cancer invasion.

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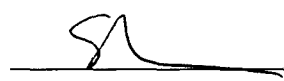

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I. INTRODUCTION

Current evidence suggests that breast carcinoma cells invade local tissues and metastasize by i) altering their cell-cell and cell-matrix interactions, ii) displaying an aberrant motile phenotype, and iii) either synthesizing, or inducing the synthesis of, proteolytic enzymes that degrade the structural barriers established by the extracellular matrix[1-3]. The complex changes in the gene program of neoplastic cells that regulate the expression of this phenotype are largely undefined, but increased interest has focused on identifying those genes that are specifically overexpressed in human breast cancer[(e.g., 3-10)]. Such information not only provides new insights into the cellular factors that control tissue-invasive behavior, but may also lead to improvements in patient diagnosis and to the more rational design of therapeutic interventions[3-10]. Consistent with this rationale, direct comparisons of the gene expression profile displayed in normal versus neoplastic breast cancer cell lines, or normal and carcinomatous breast tissues, have provided a number of novel insights into the mechanisms and processes underlying tumor progression[6-11]. Interestingly, despite the power of the analytical techniques employed for these purposes, the number of differentially expressed genes identified thus far are - at first glance - perplexingly small, despite the striking changes known to occur in cellular behavior[(e.g., 7,8)]. However, analyses of breast cancer cell lines grown *in vitro* or static tumor masses recovered from *in vivo* sites of disease may be problematic. First, comparisons between normal and neoplastic breast cancer cell lines grown atop plastic substrata *in vitro* will not recapitulate the complex interactions known to occur across the carcinoma-mesenchymal cell axis *in vivo*[1,2]. Indeed, many of the most interesting gene products that have been associated with the expression of tissue-invasive phenotypes in breast cancer tissue are synthesized by surrounding stromal cells rather than the tumor itself[2,3,10]. Secondly, while the gene expression patterns identified in tissues recovered from *in vivo* sites clearly circumvent the limitations inherent in the *in vitro* studies, only a small percentage of the cells recovered from a tumor mass at a single, fixed time point would be expected to be actively engaged in invasive behavior. Given the many similarities between developmental/tissue repair processes and malignant growth (re; the ability of cancer cells inappropriately recapitulate developmental programs associated with epithelial-mesenchymal cell transitions or repair programs associated with wound healing[12,13]), we have considered the possibility that the *in situ* induction of a synchronous matrix remodeling program in normal tissues would allow for the more efficient isolation of those gene products critical to cancer cell invasion. Indeed, recent studies have demonstrated that gene expression patterns associated with the tissue remodeling program induced during the involution of the normal lactating mammary gland bear considerable overlap with those detected in the early stages of carcinogenesis (e.g., stromeylsin-1, stromeylsin-3, urokinase-type plasminogen activator, tissue inhibitor of metalloproteinases[14-16]). Hence, we propose to use the involuting mammary gland explant model as a means to rapidly enrich for, and identify, the subset of genes that control the disassembly of the extracellular matrix in cancerous states. Furthermore, by selectively identifying the subset of gene products that encode secreted proteins in breast cancer tissue, new diagnostics as well as novel targets for therapeutic intervention can be rapidly identified.

II. BODY

In accordance with the tasks outlined in the original Statement of Work, mouse mammary gland explants were recovered from estrogen/progesterone-primed animals and cultured *in vitro* under

conditions designed to either induce lobuloalveolar development (i.e., cultured under serum-free conditions in the presence of insulin, aldosterone, hydrocortisone and prolactin as described) or involution (i.e., withdrawal of lactogenic hormones)[17]. Subsequently, glands were removed from culture after 7 days for poly(A)+ mRNA extraction (FasTrack isolation kit) and plasmid library construction. Test (i.e., involuting mammary gland tissue) and control libraries (i.e., glands undergoing lobuloalveolar gland development) were constructed using the SuperScript plasmid system (Life Technologies) and the respective cDNA ligated to pSPORT1 and pSPORT2 plasmids as described. Plasmids were then electroporated and deletions of the transformed cells plated. To isolate the differentially expressed genes, single-strand DNA (ssDNA) and biotinylated RNA were generated and hybridized[18]. The subtracted ssDNA was then converted to dsDNA to increase transformation efficiency. Following repair, the recovered DNA was electroporated into ElectroMax cells to determine the number of transforments in the subtracted library.

Prior to determining subtraction efficiency and enrichment or sequencing random colonies, we noted an oversight in the original experimental plan wherein the frequency of apoptotic cells (and hence, matrix remodeling) in the control glands undergoing lobuloalveolar development was assumed to be low. In hindsight, this assumption appeared reasonable as the whole mount analyses of the lactogenic hormone-treated glands (submitted/described in the original proposal) demonstrated significant tubulogenesis while the involuting glands actively underwent regression. However, to ensure that control/non-involuting glands were not "contaminated" with gene products associated with apoptosis/involution, control and test glands were examined for apoptotic bodies by ApoTag analysis (Oncor Corp.). In brief, mammary gland specimens were fixed and embedded as described previously[19]. Apoptotic cell nuclei were identified per the manufacturer's instructions wherein sections were treated with proteinase K, incubated with TdT and anti-digoxigonin, and color developed with dimethylaminoazobenzene and H_2O_2[19]. Sections were viewed at x440 and the number of apoptotic nuclei determined per high powered field (hpf). Surprisingly, whereas the involuting tissues displayed an expected response (i.e., a high number of apoptotic cells/hpf; >30 apoptotic cells/hpf), the control tissues likewise displayed large numbers of apoptotic cells (i.e., >20 apoptotic cells/hpf for 5 experiments). Thus, these data suggested that whereas lobuloalveolar development predominated over apoptosis in the control glands, involution programs (and their associated gene products) were likely engaged. Because a contamination of the control cDNA libraries with apoptosis-associated genes would invalidate our subtraction protocol, efforts were initiated to identify an alternate approach to obtain the required mRNA from "stable" versus "remodeling" mammary gland tissue.

While initial efforts focused on improving the *in vitro* culture system, unacceptably high rates of apoptosis continue to occur regardless of changes in the concentration of lactogenic hormones or incubation conditions (5-95% O_2)[20]. Alternatively, we posited that ideal conditions (i.e., low if not absent apoptosis in control glands with high apoptosis in involuting glands) should be more readily accessed by retrieving glands directly from control, lactating and weaned animals (i.e., omitting the *in vitro* culture step). Consequently, mammary glands were isolated from virgin, 16-d pregnant, 1-d, 5-d, and 10-day lactating animals as well as 0-d and 5-d post-weaning. Specimens were fixed and embedded as described above, and processed for apoptosis with ApoTag (results expressed as number of apoptotic cells/hpf). In groups of 5 animals, apoptosis was held at extremely low rates in virgin, pregnant and lactating animals (i.e., <0.5 cells/hpf in

virgin, pregnant and 1-10 day lactating glands). In marked contrast, apoptosis increased dramatically at 3 day post-weaning to >50 cells/hpf. Given these results, we have opted to re-construct our cDNA libraries from 5 day lactating and 3 day involuting glands. This approach should ensure that the matrix remodeling program associated with involution is compared directly to the appropriate "intact" tissue control. As such, poly(A)+ mRNA have been isolated from a pool of 5 glands (from 5 animals) and directional libraries are currently under construction (Statement of Work Tasks 1 and 2).

In Task 3, we described a method for identifying the subset of differentially expressed genes that encode secreted products. In this technique, serum-free conditioned media from involuting tissues cultured *in vitro* is used to generate polyclonal antisera which is then used to screen an expression library[21]. Despite the fact that we are no longer using the *in vitro* gland culture system to generate cDNA libraries because of unacceptable rates of apoptosis in the control glands, media collected from involuting glands can nonetheless be used for antisera generation. As such, serum-free conditioned media from involuting glands has been prepared and is ready for use as an immunogen in rabbits. Following generation of the new subtraction library, the antisera will be used to screen for apoptosis-associated secretory gene products as described in the original proposal.

III. KEY RESEARCH ACCOMPLISHMENTS

- cDNA library construction of lactating versus involuting mammary glands.
- Development of an *in vitro* model of mammary gland involution for identifying secreted matrix remodeling-associated gene products.

IV. REPORTABLE OUTCOMES

None

V. CONCLUSIONS

With the identification of suitable mammary gland tissues for isolating gene products differentially expressed during matrix-remodeling events, a model system is now in place for identifying the subset of genes that likely control the disassembly of the matrix during tumor invasion and metastasis. Furthermore, by selectively identifying those gene products that encode secreted proteins in breast cancer tissue, new diagnostics as well as novel targets for therapeutic intervention may be identified.

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